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COPII-Coated Vesicle Formation Reconstituted with Purified Coat Proteins and Chemically Defined Liposomes

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Summary

COPII vesicle formation requires only three coat assembly subunits: Sar1p, Sec13/31p, and Sec23/24p. PI 4-phosphate or PI 4,5-bisphosphate is required for the binding of these proteins to liposomes. The GTP-bound form of Sar1p recruits Sec23/24p to the liposomes as well as to the ER membranes, and this Sar1p-Sec23/24p complex is required for the binding of Sec13/31p. Ultrastructural analysis shows that the binding of COPII coat proteins to liposomes results in coated patches, coated buds, and coated vesicles of 50–90 nm in diameter. Budding proceeds without rupture of the donor liposome or vesicle product. These observations suggest that the assembly of the COPII coat on the ER occurs by a sequential binding of coat proteins to specific lipids and that this assembly promotes the budding of COPII-coated vesicles.

Introduction

Transport between the membrane compartments of the secretory pathway in eukaryotic cells is mediated by vesicles, which are produced by a budding mechanism involving coat proteins that capture specific cargo molecules into coated vesicle intermediates (for reviews, see Rothman and Wieland, 1996; Schekman and Orci, 1996). To date, three classes of coated vesicles have been characterized: clathrin-coated vesicles mediate transfer among the plasma membrane, endocytic, and *trans*-Golgi compartments (Robinson, 1994); COPI-coated vesicles mediate intra-Golgi and endoplasmic reticulum (ER)-Golgi bidirectional transport (Rothman and Wieland, 1996; Orci et al., 1997); and COPII-coated vesicles mediate anterograde transport from the ER to the Golgi apparatus (Bednarek et al., 1996; Schekman and Orci, 1996).

Although coated transport vesicles have similar sizes (60–90 nm), the soluble factors that comprise the coats are different. The clathrin coat contains three components: two different complexes of coat proteins (clathrin triskelion and adapter complex) and a small GTP-binding protein, ADP-ribosylation factor (ARF) (Robinson, 1994; Rothman and Wieland, 1996). COPI contains two separable constituents: the coatomer complex and ARF (Orci et al., 1993a; Rothman and Wieland, 1996; Schekman and Orci, 1996). COPII consists of three parts: two coat protein complexes (Sec23/24p complex and Sec13/31p complex) (Hicke et al., 1992; Salama et al. 1993; Schekman and Orci, 1996) and one small GTP-binding protein, Sar1p (Nakano and Muramatsu, 1989; Barlowe et al., 1993).

COPII vesicles are created by the concerted action of subunits of the coat that interact with each other and with specific proteins in the ER. Sar1p, Sec23p, and Sec24p appear to serve more than simply structural roles. For example, Sec23p acts as a GTPase-activating protein specific for Sar1p that allows Sar1p-GDP to be released during or shortly after completion of the budding cycle (Yoshihisa et al., 1993). In addition, the packaging of transported proteins into forming vesicles appears to require the combined action of Sar1p and Sec23/24p, which form a cargo recruitment complex that discriminates between transported and ER resident proteins (Kuehn et al., 1998).

Two other ER proteins, Sec12p and Sec16p, serve additional essential roles in the budding cycle. Sec12p contains an N-terminal, cytoplasmically exposed domain that facilitates nucleotide exchange on Sar1p (Nakano et al., 1988; d'Enfert et al., 1991; Barlowe and Schekman, 1993). Localization of Sec12p ensures that Sar1p-mediated budding is largely restricted to the ER (Sato et al., 1996). Sec16p shows numerous genetic and physical interactions with Sec12p and with subunits of COPII (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990, Espenshade et al., 1995; Gimeno et al., 1996; Shaywitz et al., 1997). The protein can be detected in isolated COPII vesicles, yet it is not among the soluble peripheral components that can be readily removed from ER membranes and added back to reconstitute vesicle formation. Instead, Sec16p may remain firmly associated with the ER, where it may organize budding sites and facilitate capture of integral membrane proteins into COPII vesicles (Campbell and Schekman, 1997).

Because no other genes have been implicated in COPII vesicle biogenesis, we developed a biochemical approach to identify membrane components essential for the recruitment of coat subunits to the membrane. Our approach was based on the assumption that a reconstituted proteoliposome or possibly a synthetic liposome could be created that would provide a favorable binding site to nucleate the assembly of the COPII coat and of COPII vesicles. We report here that a simple closed bilayer containing acidic and neutral phospholipids satisfies the requirement for a membrane surface in the budding of coated vesicles.

Results

Binding of COPII Coat Proteins to the ER

Various combinations of COPII proteins were incubated with ER-enriched microsomal membranes that had been

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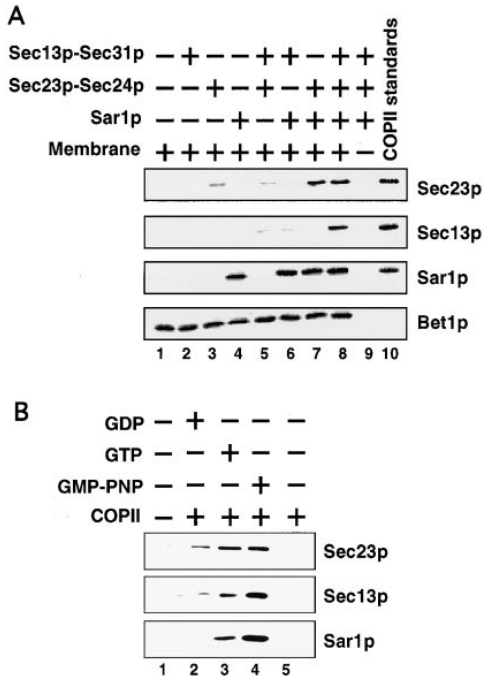

Figure 1. Binding of COPII Proteins to Urea-Washed Microsomes

(A) Various combinations of Sec13/31p (0.5 μg), Sec23/24p (0.4 μg), and Sar1p (0.3 μg) were tested for their ability to associate with urea-washed microsomes in the presence of 0.1 mM GMP-PNP in a 50 μl reaction. Membrane-associated proteins were detected by quantitative immunoblot analysis. Bet1p was used as an internal standard for the recovery of microsomes. Lane 10 contained 1/10 of the COPII proteins used in the assay.

(B) Nucleotide-dependence of the binding of COPII proteins to urea-washed microsomes. Binding reactions were performed in the presence of 0.1 mM GDP, GTP, or GMP-PNP as in (A). COPII, all COPII components.

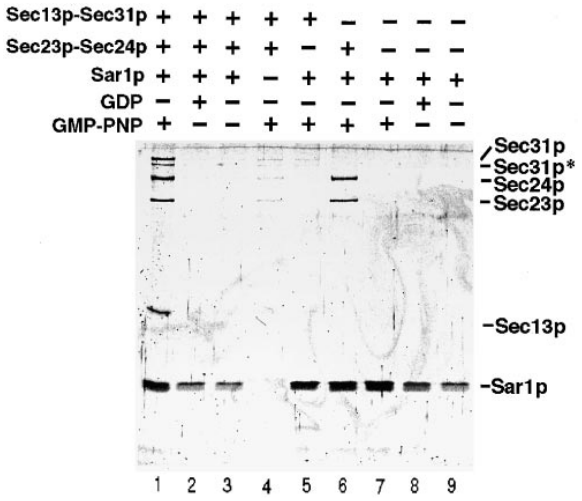

Figure 2. Binding of COPII Proteins to the Liposomes Made from Microsomal Lipids

Various combinations of Sec13/31p (2 μg), Sec23/24p (1.7 μg), Sar1p (1.6 μg), GDP, or GMP-PNP (0.1 mM) were incubated with liposomes made of microsomal lipids in a 100 μl reaction. Liposome-associated proteins were resolved by SDS-PAGE and stained by SYPRO Red. Sec31p* indicates a degradation product of Sec31p that is present in our Sec13/31p preparation.

washed with urea, and coat protein–ER complexes were separated from unbound proteins by centrifugation (Figure 1A). In the presence of GMP-PNP, Sar1p exhibited a comparable level of binding to membranes in the presence or absence of other COPII components. Binding of Sec23p was enhanced in the presence of Sar1p. Binding of Sec13p required the presence of Sar1p and the Sec23/24p complex (Figure 1A, lane 8). Recruitment of Sec24p and Sec31p was coincident with Sec23p and Sec13p, respectively (data not shown). The binding of COPII components was detected in the presence of GTP, although GMP-PNP afforded a higher level of binding (Figure 1B). GDP did not substitute for GMP-PNP, indicating that the recruitment of Sar1p to the ER membrane required guanine nucleoside triphosphate.

Binding of COPII Proteins to Liposomes Made from Microsomal Lipids

In order to isolate membrane components responsible for the binding of COPII to the ER, we solubilized microsomal membranes with octylglucoside. The separated protein fractions were incorporated into proteoliposomes formed using lipids prepared by organic solvent extraction from microsomes. COPII proteins were added and bound complexes were analyzed by buoyant density flotation. Although proteoliposomes bound COPII in quantities similar to native ER membrane, we were unable to correlate the binding activity with any membrane protein fraction (data not shown).

We next examined the possibility that the COPII binding observed with proteoliposomes was mediated by a direct interaction between coat proteins and lipids. Liposomes formed from microsomal lipids were mixed with COPII proteins in the presence or absence of guanine nucleotide. Protein-liposome complexes were then isolated by flotation on sucrose density gradients. Sar1p bound to liposomes in the absence of nucleotide or in the presence of GDP; however, binding was enhanced approximately 2-fold by GMP-PNP (Figure 2). In contrast, binding of Sec23/24p and Sec13/31p to liposomes was only observed in the presence of GMP-PNP (Figure 2, lanes 1 and 2). Unlike native ER, liposomes did not support stable coat protein binding in the presence of GTP (not shown). Incubation of liposomes with various combinations of coat subunits showed that the binding of Sec23p and Sec24p required Sar1p and GMP-PNP (Figure 2, lane 6) and that both of these protein fractions were required for the binding of Sec13p and Sec31p (Figure 2, lane 1). These results indicated that lipids comprise the factor required to confer nucleotide-dependent recruitment of COPII proteins to membrane. An additional component, possibly a membrane protein, may be necessary to prevent GTP hydrolysis and to stabilize Sar1p-GTP for recruitment of the other coat subunits. In the rest of this investigation we have used Sar1p equilibrated with GMP-PNP to retain the triphosphate and to bypass the requirement for Sec12p-mediated nucleotide exchange.

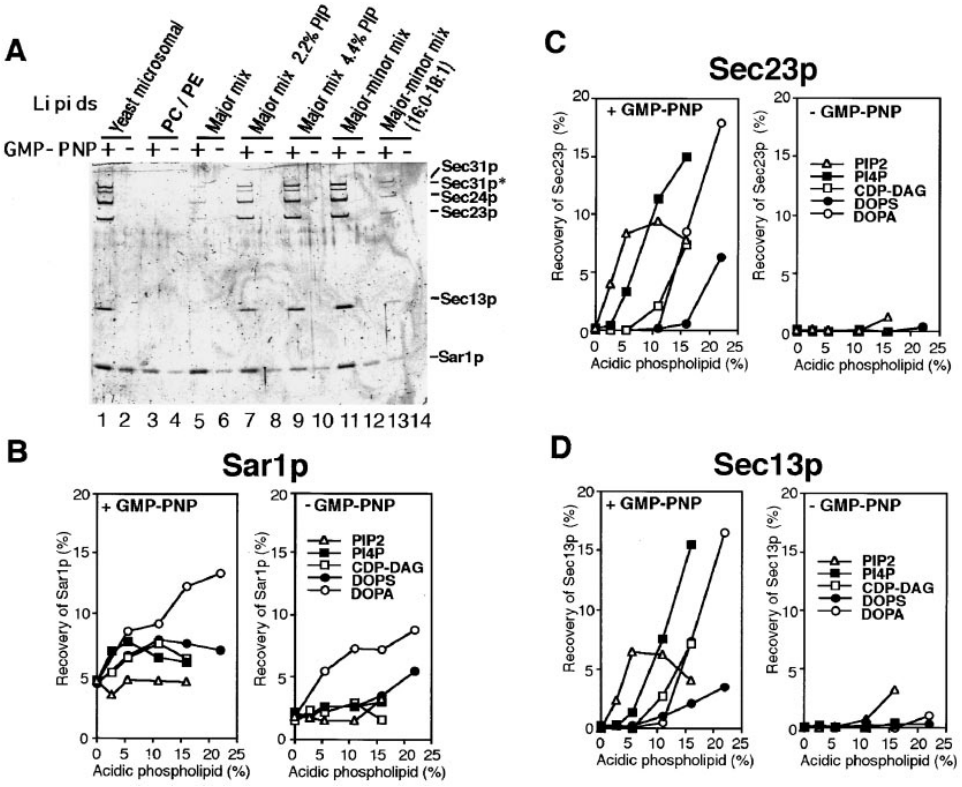

Figure 3. Binding of COPII Proteins to the Liposomes Made from Pure Phospholipids

(A) Liposomes (corresponds to 9 μg phospholipids) composed of various combinations of phospholipids were tested for the binding of COPII proteins in the presence or absence of 0.1 mM GMP-PNP in a 100 μl reaction. Liposome-bound proteins were analyzed as described in the legend of Figure 2. PC/PE: 53 mol% DOPC, 47 mol% DOPE. Major mix: 53 mol% DOPC, 23 mol% DOPE, 11 mol% PI (from soybean), 8 mol% DOPS, 5 mol% DOPA. Major mix 2.2% PIP: PI4P was substituted for 2.2 mol% of PI in major mix. Major mix 4.4% PIP: PI4P was substituted for 4.4 mol% of PI in major mix. Major-minor mix: 51 mol% DOPC, 23 mol% DOPE, 9 mol% PI, 8 mol% DOPS, 5 mol% DOPA, 2.2 mol% PI4P, 0.8 mol% PIP_2, 2 mol% CDP-DAG. Major-minor mix (16:0–18:1): 1-linoleyl-2-oleoyl-derivative of PC, PE, PS, and PA was substituted for DOPC, DOPE, DOPS, and DOPA in major-minor mix, respectively.

(B–D) Titration of acidic phospholipids in PC/PE liposomes. Various amounts of one of the acidic phospholipids were substituted for DOPC in PC/PE liposomes. The efficiency of binding of COPII proteins (percentage of the input of each protein recovered in the floated fraction) in the presence (+GMP-PNP) and absence (−GMP-PNP) of 0.1 mM GMP-PNP was analyzed. (B), Sar1p. (C), Sec23p. (D), Sec13p.

Phospholipid Requirement for Binding of COPII Proteins to Liposomes

To identify the individual lipids required for the binding of COPII components to a membrane, we made liposomes from purified phospholipids. As a starting point, liposomes were made from a mixture with a composition similar to microsomal membranes from yeast (Zinser and Daum, 1995): 53 mol% dioleolylphosphatidylcholine (DOPC), 23 mol% dioleoylphosphatidylethanolamine (DOPE), 8 mol% dioleoylphosphatidylserine (DOPS), 5 mol% dioleoylphosphatidic acid (DOPA), and 11 mol% phosphatidylinositol (PI) from soybean (major mix). We used dioleoyl-derivatives of PC, PE, PS, and PA because most of the phospholipids in yeast contain monounsaturated fatty acyl chains in the sn-1 and sn-2 positions (Wagner and Paltauf, 1994).

Nearly normal levels of Sar1p bound to these liposomes and the association was stimulated by GMP-PNP (Figure 3A, lanes 5 and 6). In contrast, only weak binding of Sec23/24p and Sec13/31p was detected in the presence of GMP-PNP. The failure to recruit Sec23/24p and Sec13/31p to liposomes made of DOPC and DOPE (Figure 3A, lanes 3 and 4) suggested that acidic phospholipids in the major mix contributed to the binding. When a portion of PI in the major mix was replaced with phosphatidylinositol 4-phosphate (PI4P), the GMP-PNP- and Sar1p-dependent binding of Sec23/24p and Sec13/31p was dramatically increased and was comparable to the binding to liposomes made from microsomal lipids (Figure 3A, lanes 1, 2, and 7–10). Inclusion of phosphatidylinositol 4,5-diphosphate (PIP_2) and CDP-diacylglycerol (CDP-DAG) in a lipid mixture that contained PI4P further enhanced coat protein binding (Figure 3A, lanes 11 and 12). Under these conditions (major-minor mix), about 24% of the Sec13p, Sec23p, Sec24p, and Sec31p and 50% of the lipids were recovered in the same fraction (data not shown). The inclusion of PI4P, PIP_2, and CDP-DAG did not significantly enhance the binding of Sar1p to the liposomes. Thus, Sar1p-GMP-PNP may bind membranes through neutral phospholipid, and Sec23/24p and Sec13/31p may bind first to Sar1p-GMP-PNP and then be stabilized on membranes through an independent interaction with anionic phospholipid.

Addition of lysophospholipids (10% by weight) or oleic acid (10% by weight), which may increase the fluidity of membranes, increased the nucleotide-independent binding of Sar1p to the liposomes without affecting the efficiency of binding of other proteins. In contrast, inclusion of other lipids individually or in combination, such as ergosterol (up to 25% by weight), ceramide, or dioleoylglycerol, or replacement of DOPE with dioleoyl-[N-(7-nitrobenz-2-oxa-1,3-diazoyl-4yl)]-sn-glycero-phosphoethanolamine (NBD-PE, 2 mol%) or of DOPC with 1-hexadecanoyl-2-[N-nitrobenz-2-oxa-1,3-diazoyl-4yl]-sn-glycero-3-phosphocholine (NBD-PC, 2 mol%) did not alter the binding efficiency significantly (data not shown).

We explored the influence of lipid unsaturation on the binding of COPII components using liposomes containing 1-palmitoyl-2-oleoyl derivatives of PA, PC, PE, and PS (containing one saturated and one unsaturated fatty acyl chain) instead of the dioleoyl derivatives of the phospholipids (containing two unsaturated acyl chain) in the major-minor mix. These liposomes showed a lower capacity to bind COPII protein than liposomes formed with two unsaturated fatty acyl chains (Figure 3A, lanes 13 and 14). Much of this reduction can be attributed to less-efficient binding of Sar1p. These results suggest that the fluidity of the lipid phase influences efficient binding of COPII components, especially of Sar1p, to the liposomes.

To evaluate the contribution of each acidic phospholipid to the binding of Sec23/24p and Sec13/31p, we made liposomes containing an increased concentration of one of the acidic phospholipids with a corresponding decrease in the concentration of DOPC and a fixed concentration (31 mol%) of DOPE. Except for DOPA, the increase in concentration of acidic phospholipids did not enhance the binding of Sar1p to the liposomes significantly (Figure 3B). However, acidic phospholipids increased the binding of Sec23p and Sec13p in a dose-dependent manner (Figures 3C and 3D). A quantitatively similar behavior was found for Sec24p and Sec31p.

Among the acidic phospholipids tested, PIP_2 supported the binding of Sec23p and Sec13p at the lowest concentration (2.8 mol%). However, the recruitment of Sec23p and Sec13p to liposomes containing PIP_2 was limited, and binding plateaued at a relatively low concentration of PIP_2 (5.5 mol%). Higher concentrations of PIP_2 allowed GMP-PNP-independent binding of Sec23p and Sec13p to the liposomes (Figures 3C and 3D, right panel). In contrast to PIP_2, an increase in the amount of PI4P enhanced GMP-PNP-dependent binding of Sec23p and Sec13p to the liposomes, and this effect was not saturated at the concentrations tested. Even at high concentrations, PI4P did not cause nucleotide-independent binding of Sec23p and Sec13p to the liposomes. Among the other acidic phospholipids, DOPA allowed efficient binding of Sec23p and Sec13p only at unphysiologically high concentrations (15–22 mol%) (Figures 3C and 3D). DOPS afforded poor recruitment of Sec23p and Sec13p even at high concentration (22 mol%), and PI supported no binding at all (data not shown). These observations suggested that acidic phospholipids, particularly PIP_2 and PI4P, are required for the efficient recruitment of Sec23/24p and Sec13/31p to the lipid bilayer in a GMP-PNP- and Sar1p-dependent manner.

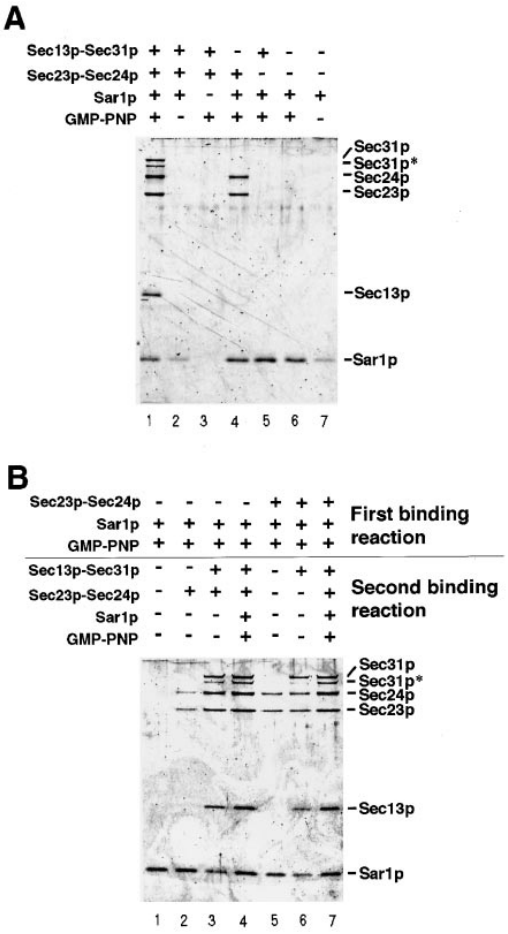

Figure 4. Sequential Binding of COPII Proteins to the Liposomes

(A) Protein requirement for the binding to the liposomes. Various combinations of COPII proteins were incubated with liposomes made from the major-minor mix and the binding was analyzed.

(B) Sequential binding. Liposomes made from the major-minor mix were incubated with Sar1p and GMP-PNP or Sar1p, Sec23/24p, and GMP-PNP and separated from nonbound proteins by flotation centrifugation (first binding reaction). Recovered liposomes (with protein) were subjected to a second-round binding reaction with various combinations of COPII proteins in the presence or absence of GMP-PNP (second binding reaction). Proteins recovered in the second binding reaction were analyzed.

Although the major-minor mix did not contain a high concentration of any one of the acidic phospholipids, liposomes made from this mixture recruited Sec23/24p and Sec13/31p efficiently (Figure 3A). This strong binding could result from a synergy among different acidic phospholipids. For example, a mixture including 5.5 mol% DOPA and 16.5 mol% DOPS in DOPC/DOPE liposomes afforded binding similar to liposomes made of 22 mol% DOPA, 47 mol% DOPC, and 31 mol% DOPE. However, liposomes containing either 5.5 mol% of DOPA or 16.5 mol% of DOPS alone in DOPE/DOPC liposomes produced almost no binding of Sec23/24p and Sec13/31p (Figures 3C and 3D; data not shown).

Sequential Binding of COPII Proteins to Liposomes

To investigate whether the synthetic liposomes recapitulated the COPII binding properties of the ER, we incubated various combinations of coat proteins with liposomes made of the major-minor mix and analyzed isolated protein–liposome complexes (Figure 4A). The binding of Sar1p to the liposomes was stimulated by GMP-PNP. Likewise, binding of Sec23p and Sec24p was strictly dependent on Sar1p and GMP-PNP (Figure 4A, lanes 1 and 4), and Sec13p and Sec31p were recruited only in the presence of the other COPII components and GMP-PNP (Figure 4A, lane 1).

Two-step binding reactions were carried out to address whether the recruitment of COPII components to the liposomes occurred sequentially. We included fluorescent phospholipids (2 mol% of NBD-PE and 2 mol% of NBD-PC) in the major-minor mix to monitor the recovery of lipids during the preparation of the samples. In a first stage, Sar1p or Sar1p and Sec23/24p were incubated with liposomes in the presence of GMP-PNP (first binding reaction). Protein-bound liposomes were separated by centrifugation from free proteins and GMP-PNP. The floated liposomes were further incubated either with Sec23/24p, Sec13/31p, or Sec23/24p and Sec13/31p or with all of these proteins and GMP-PNP (second binding reaction). Liposomes were separated again from free proteins, and bound complexes were analyzed (Figure 4B). The recovery of lipids, monitored by the fluorescence of NBD, was almost identical in each sample during the two-step binding.

Sec23p and Sec24p bound to the liposomes that had been incubated with Sar1p and GMP-PNP in the first binding reaction. The binding of Sec23p and Sec24p was enhanced in the presence of Sec13/31p in the second binding reaction (Figure 4B, lanes 2 and 3). Sec13p and Sec31p only bound to liposomes that had been incubated with Sar1p, GMP-PNP, and Sec23/24p (Figure 4B, lane 6). These results indicate that the binding of Sar1p, Sec23/24p, and Sec13/31p to the liposomes occurs sequentially and suggest that the binding of Sec13/31p stabilizes the recovery of Sec23/24p.

Ultrastructure of Liposomes with COPII Proteins

We next investigated the morphological consequence of COPII binding to liposomes. Since only about 5% of the lipids participated in the binding of COPII proteins in the conditions used above, we increased the coat protein concentration to enhance the visual detection of intermediates. A higher concentration of COPII proteins (2.5 $\times$ normal) increased the percentage of liposomes that participate in binding without changing the nucleotide dependency (see below). We included ergosterol in the lipid mixture (20% by weight) to enhance the preservation of membrane ultrastructure (data not shown).

Liposomes made by extrusion through a polycarbonate filter (400 nm pore size) contained many uni- and multilamellar structures (Figure 5A). The average size of these liposomes was approximately 300 nm in diameter, with fewer than 5% of the profiles smaller than 90 nm (Table 1). After incubation with COPII proteins and GMP-PNP, many liposomes showed coated surfaces (Figure 5B). Coats often clustered to form a coated patch with coated and noncoated surfaces clearly separated. Coated surfaces were also observed to form buds and tubules and numerous coated vesicles of around 60 nm in diameter. Quantitative analysis indicated that the incubation of liposomes with COPII proteins and GMP-PNP increased the number of small vesicles (diameter $<$90 nm; Table 1), most of which were coated (Figure 5B).

Coated membrane, coated bud, and vesicle formation required GMP-PNP. However, in the absence of nucleotide, solid mesh-like spherical aggregates of about 90 nm were seen (Figure 5C). These aggregates were also found in samples that contained COPII proteins without liposomes (not shown). In the presence of GMP-PNP, Sar1p, and Sec23/24p, but in the absence of Sec13/31p, no liposomes showed the coat structure (not shown). These observations indicate that COPII proteins bound to the liposomes form coats that gives rise to coated buds and vesicles. As with native ER membranes, the appearance of a coat on liposomes requires the full set of COPII proteins.

Isolation and Characterization of COPII-Coated Vesicles Derived from Liposomes

We centrifuged the products of a synthetic budding reaction on a linear sucrose-density gradient to separate the coated vesicles from noncoated liposomes (Figure 6A). The sedimentation of lipids was monitored by measuring the fluorescence of NBD-phospholipids. Major phospholipids in the mixture, such as DOPE, DOPC, DOPS, and PI, sedimented coincidentally to the NBD fluorescence.

When large liposomes, prepared by extrusion with a 400 nm filter, were incubated with a high concentration of COPII proteins (5 $\times$ normal) and GMP-PNP, we detected three peaks of lipid in the density gradient (Figure 6A). GMP-PNP-dependent sedimentation of lipids that migrated to two different peaks (fractions 7 and 13, corresponding to 17% and 31% sucrose, respectively) indicated that the density shift was the result of the binding of COPII proteins to the liposomes. In contrast, small liposomes (made by extrusion with a 50 nm filter) incubated with excess COPII proteins (5 $\times$ normal), and GMP-PNP migrated as a single peak corresponding to 25% sucrose (Figure 6A). The density of COPII-coated vesicles formed from intact ER (42% sucrose) exceeded that of each of the synthetic species.

The recovery of the lipids in the high-density peak (fractions 11–15) depended on the concentration of COPII proteins. At a low concentration of COPII proteins (1 $\times$ COPII, Figure 6B) similar to that used for the budding of COPII vesicles from intact ER, we did not detect significant lipid migrating in fractions 11–15 (Figure 6B). The recovery of lipids in the low-density peak (fractions 5–10) increased significantly when the concentration of COPII proteins was increased to a moderate concentration (2.5$\times$, Figure 6B), whereas higher concentrations of COPII proteins were required to yield lipids in the high-density peak (fractions 11–15). Prolonged incubation or a higher temperature during the incubation also increased the recovery of lipids in the high-density peak

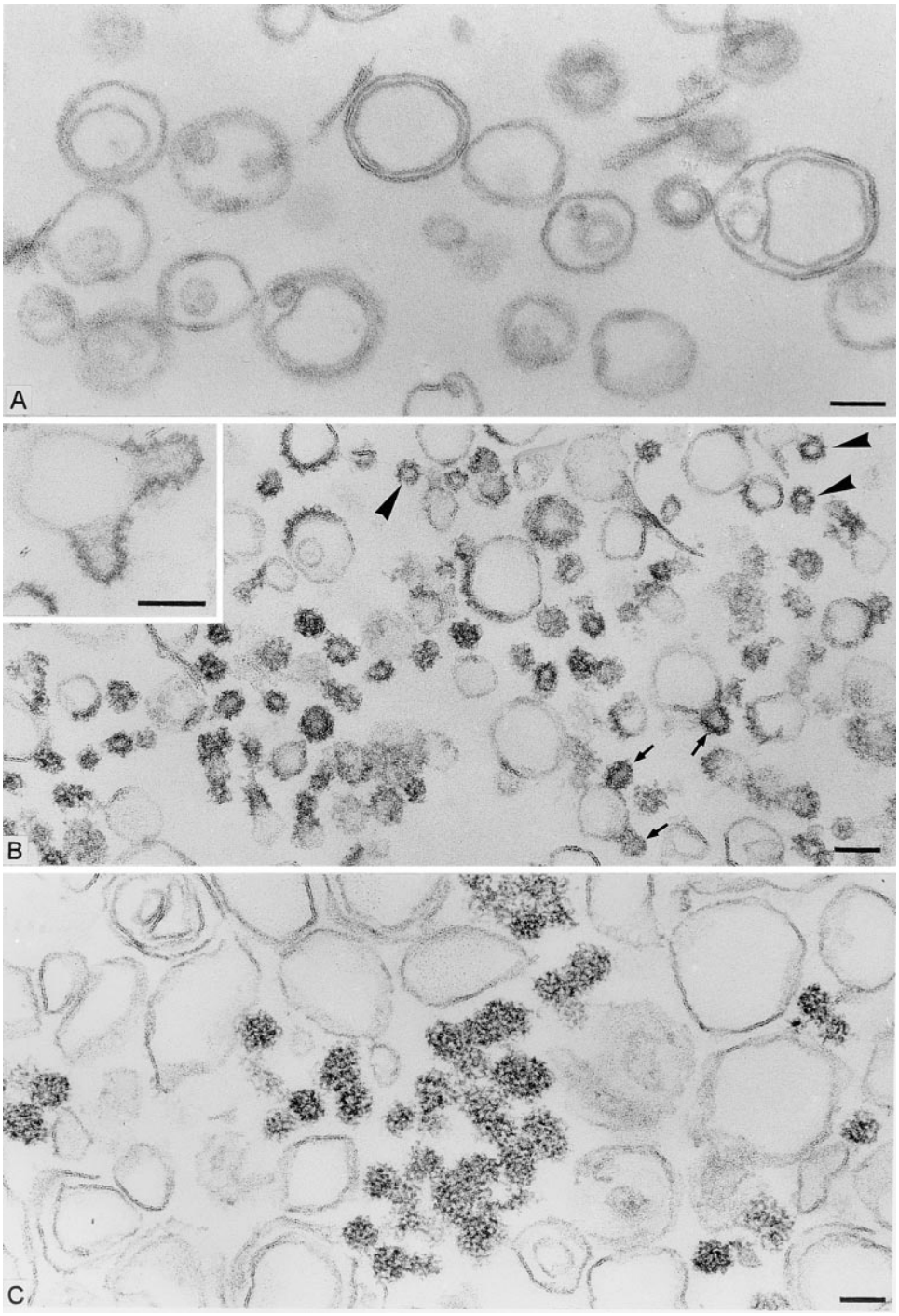

Figure 5. Morphology of Liposomes Incubated with COPII Proteins and GMP-PNP

(A) Liposomes used for the binding experiment before incubation. The circular bilayer profiles (large and small) appear smooth on their outer surfaces.

(B) Liposomes were incubated with Sec13/31p (12.5 μg), Sec23/24p (10.6 μg), and Sar1p (10 μg) in the presence of 0.1 mM GMP-PNP for 15 min on ice in a 250 μl reaction. Distinct coat segments are present on the outer aspect of large and small circular liposome profiles. On some large profiles, coat binding determines a budding process from the liposome bilayer (arrows and insert). Free vesicle-like coated liposomes are indicated by arrowheads.

(C) Liposomes were incubated with Sec13/31p (12.5 μg), Sec23/24p (10.6 μg), and Sar1p (10 μg) in the absence of 0.1 mM GMP-PNP for 15 min on ice in a 250 μl reaction. Mesh-like spherical aggregates of electron-dense coat material are present between the smooth-surfaced circular profiles of liposomes.

The bars represent 100 nm.

Table 1. Size Distribution of Liposomes before and after Incubation with COPII Coat Proteins and GMP-PNP

	Incubation Condition	Diameter (nm) (Mean ± SD)	Liposomes or Vesicles <90 nm (% of Profiles)	N
Experiment 1				
	Before incubation	286 ± 142	2	240
	Sar1p, Sec23/24p, Sec13/31p, GMP-PNP, on ice 15 min	158 ± 82	37	240
	Sar1p, Sec23/24p, Sec13/31 p, on ice 15 min	370 ± 177	5	240
	Sar1p, Sec23/24p, GMP-PNP, on ice 15 min	295 ± 165	7	240
Experiment 2				
	Before incubation	332 ± 178	5	120
	Sar1p, Sec23/24p, Sec13/31p, GMP-PNP, on ice 15 min	149 ± 66	38	120
	Sar1p, Sec23/24p, Sec13/31p, on ice 15 min	311 ± 147	3	120
	Sar1p, Sec23/24p, GMP-PNP, on ice 15 min	270 ± 144	9	120

Incubation of liposomes with COPII proteins and GMP-PNP was carried out as described in the legend of Figure 5.

with a corresponding decrease in the low-density peak (Figure 6B). However, the same treatment did not cause a density shift of preformed liposomes (50 nm) that had been incubated with excess level of COPII proteins and GMP-PNP (data not shown). These observations indicate that the formation of the dense species from 400 nm liposomes is not a simple binding of coat proteins to small liposomes that are present or form spontaneously in the liposome fraction.

Ultrastructural analysis clearly demonstrated that the high density fraction contained heavily coated small liposomes that resembled COPII-coated vesicles derived from the ER (Figures 7B–7D). However, the size variation of these liposomes was larger than that of the COPII vesicles from the ER (Table 2; Figures 7B–7D). The average size of the synthetic coated vesicles varied with conditions of incubation, and higher concentrations of coat proteins resulted in larger coated vesicles (Table 2). In contrast to the high-density liposomes, the low-density fraction contained large liposomes that were lightly or partially coated (Figure 7A). The substructure of the coat on small vesicle-like liposomes is shown in Figures 7E and 7F. Based on these observations, we conclude that at low concentrations COPII proteins coat the surface of any appropriate membrane. However, at high concentrations the pure proteins may cluster to deform the surface of a large liposome, producing coated buds and vesicles.

The stoichiometry of coat subunits in native and synthetic COPII vesicle were compared. Synthetic COPII vesicles recovered from the high-density fraction (Figure 6A) were further purified by gel filtration. In an average of two experiments, native COPII vesicles formed in the presence of GMP-PNP had approximately three subunits of Sar1p for each of the Sec protein subunits, which were themselves roughly equimolar. Synthetic COPII vesicles contained approximately two subunits of Sar1p, Sec13p, and Sec31p for each copy of Sec23p and Sec24p. Thus, the presence of membrane proteins within the ER may influence the exact ratio of COPII subunits that create a coated vesicle.

Integrity of Liposomes and Liposome-Derived COPII vesicles

We incorporated a water-soluble and membrane-impermeable fluorescent dye (Hydroxypyrene-1,3,6-trisulfonic acid; HPTS) into liposomes to monitor the transfer of luminal contents into synthetic COPII vesicles. Liposomes containing HPTS were incubated with COPII proteins and GMP-PNP in the presence or absence of a membrane-impermeable fluorescence-quenching reagent (p-xylene-bis-pyridinium bromide; DPX). The presence of these reagents had no effect on the binding of COPII coat proteins to liposomes (data not shown). After a 30 min incubation, samples not containing DPX were supplemented with the quencher, and, finally, samples were treated with Triton X-100 to dissolve all membranes. Although COPII proteins alone weakly quenched the fluorescence of HPTS, no decline was detected on addition of coat proteins and GMP-PNP under conditions of active membrane budding (Figure 8A). Complete quenching occurred when the luminal and extravesicular fluid mixed on addition of detergent. We conclude that membrane rupture and resealing does not occur during the budding reaction.

We next examined the recovery of luminal content in isolated synthetic COPII vesicles. Density gradient fractionation was conducted on samples prepared from HPTS-containing liposomes. Fluorescent vesicles were

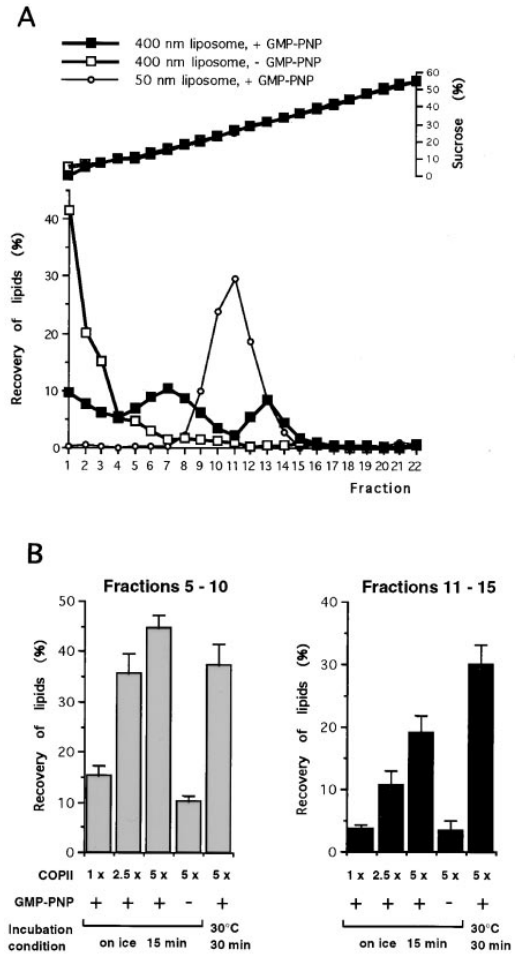

Figure 6. Formation of Coated Vesicles Requires a Higher Concentration of COPII Proteins

(A) Distribution of lipids on a sucrose-density gradient after binding reaction and centrifugation. Liposomes made by extrusion through filters with 400 nm or 50 nm pore sizes, containing 9 μg of phospholipids and 2.25 μg of ergosterol, were used for the binding reaction with 10 μg Sec13/31p, 8.5 μg Sec23/24p, and 8 μg Sar1p with or without GMP-PNP in a 100 μl reaction. After incubation on ice for 15 min, the mixture was loaded on a sucrose gradient and centrifuged for 16 hr. The recovery of lipids was monitored by the fluorescence of NBD-phospholipids. The upper panel indicates the concentration of sucrose after centrifugaton.

(B) Formation of the high-density lipid peak depends on the concentration of COPII proteins and incubation conditions. Binding reactions and separation on a sucrose gradient were carried out as in (A) in the presence or absence of GMP-PNP and various concentrations of COPII proteins using different conditions of incubation. Recovery NBD fluorescence in the fractions 5–10 and 11–15 is shown. The error bar indicates the standard error of the recovery of at least three independent experiments. 1 × COPII: 20 μg/ml Sec13/31p, 17 μg/ml Sec23/24p, 16 μg/ml Sar1p; 2.5 × COPII: 50 μg/ml Sec13/31p, 43 μg/ml Sec23/24p, 40 μg/ml Sar1p; 5 × COPII: 100 μg/ml Sec13/31p, 85 μg/ml Sec23/24p, 80 μg/ml Sar1p.

isolated at the normal high-density position, dependent on COPII proteins and GMP-PNP. No significant difference in the recovery of fluorescence was detected in synthetic COPII vesicles isolated from budding reactions conducted in the presence or absence of DPX (Figure 8B). However, as in the unfractionated sample, addition of Triton X-100 and DPX resulted in a quench to background fluorescence. We conclude that budding of synthetic COPII vesicles occurs by a mechanism that preserves the integrity of donor membrane and budded vesicle product.

Discussion

Vesicle Morphogenesis Governed by the COPII Coat

Three cytosolic and peripheral membrane proteins (Sar1p, Sec23/24p, and Sec13/31p) are necessary and sufficient to form functional COPII vesicles from isolated ER membrane fractions. What role do membrane proteins and lipids play in this process? Only one integral membrane protein, Sec12p, is known to be required for this budding event. Sec12p serves to initiate and localize vesicle budding by facilitating nucleotide exchange on the GTP-binding protein Sar1p. In an effort to identify additional membrane components necessary for COPII assembly, we bypassed the requirement for Sec12p in vitro using recombinant Sar1p equilibrated with the nonhydrolyzable analog GMP-PNP.

A sequential recruitment of Sar1p-GMP-PNP, Sec23/24p, and Sec13/31p was established for native ER membranes and then reproduced with synthetic membrane reconstituted by dialysis of detergent-solubilized membrane fractions. The same binding parameters were recapitulated with liposomes formed from a crude yeast lipid fraction or from pure, commercially available phospholipids. We conclude that the membrane recruitment of the COPII ensemble does not depend on any membrane protein.

Morphological inspection of liposome-COPII mixtures revealed the formation of coated membrane surfaces. In addition, the coat created or stabilized numerous buds and small vesicles. Thus, the formation of the COPII coat and the appearance of coated buds and vesicles depends strictly on the purified proteins and is independent of other peripheral or integral membrane proteins. This assertion is consistent with and extends considerably our conclusion that COPII vesicle formation does not require secretory or membrane cargo protein (Yeung et al., 1995). Likewise, these results extend the conclusion of Orci et al. (1993b) that coatomer and ARF comprise the essential and rate-limiting components of COPI vesicle formation at the Golgi complex. Incubation of our liposome formulation with coatomer, mutant ARF, and GTPγS results in a similar level of synthetic COPI-coated buds and vesicles (A. Spang, S. H., K. M., R. S., and L. O., unpublished data).

Synthetic COPII vesicles detected in unfractionated samples or after isolation on a density gradient are similar in size and coat thickness to those formed from ER membranes. These comparisons strengthen the notion that the coat subunits are the principal determinant of vesicle morphogenesis. Similar conclusions may be drawn for clathrin, which forms uniformly sized empty cages at high protein concentration in the absence of membrane (Keen et al., 1979).

Certain other aspects of the synthetic budding reaction clearly do not reproduce the physiologic event. The ratio of Sar1p to the other coat subunits was somewhat different in synthetic and native COPII vesicles. Optimum budding with liposomes required a higher level of

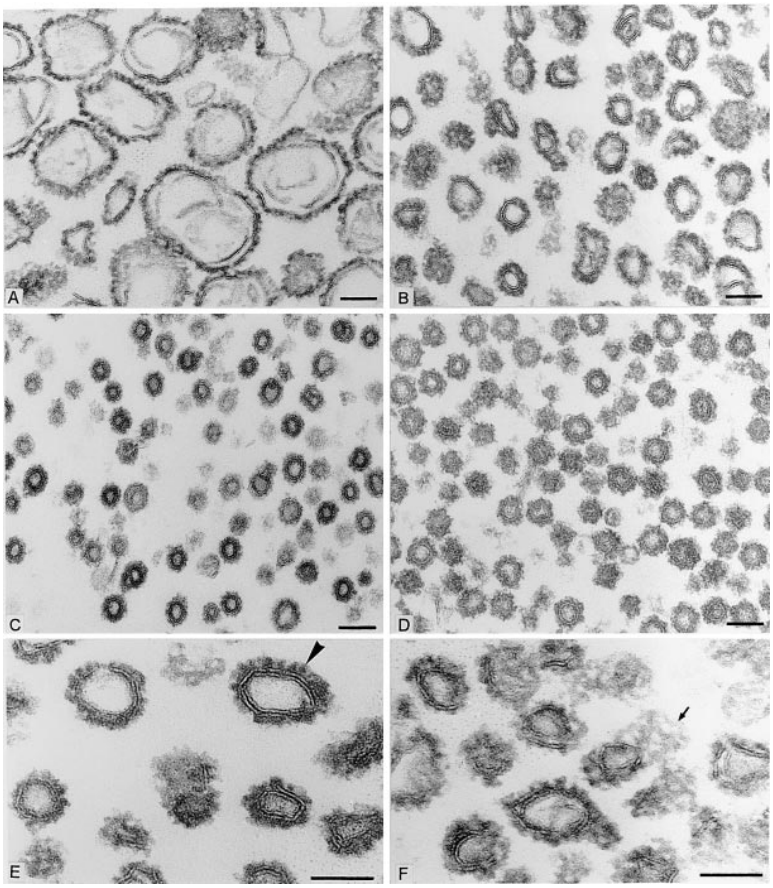

Figure 7. Morphology of COPII-Coated Liposomes after Separation by Centrifugation

(A) View of the liposomes in fractions 6 and 7 from the experiment described in Figure 6A. The field shows a population of large circular profiles (mean diameter 138 nm ± 46 SD) with a distinct outer coating visible on most of their periphery.

(B) View of the liposomes in fractions 12 and 13 of the experiment described in Figure 6A. COPII concentration = 5×. The field shows a population of relatively small circular coated vesicular profiles.

(C) Field of small coated vesicular profiles from fractions 12 and 13. COPII concentration = 2.5×. See Table 2 for the respective sizes of liposomes in (B) and (C).

(D) Field of native COPII-coated vesicles isolated from the ER.

(E and F) High magnification of coated liposomes from fractions 12 and 13. The various sectioning planes allow one to visualize the spike of the coat in equatorial sections (arrowhead) and the sieve-like pattern given by the spikes in tangential sections (arrow).

The bars represent 100 nm.

COPII proteins (2.5- to 5-fold greater) than necessary to form vesicles from intact ER membranes.

We considered the possibility that high levels of COPII proteins may trap and allow the visualization of buds and vesicles that form spontaneously from liposomes rather than actively deforming the membrane to create these profiles. Two arguments favor an active rather than passive role for the coat. First, quantitative evaluation of small vesicles detected in unfractionated samples showed a 6- to 8-fold increase in number dependent on the presence of COPII (Table 1). A second important consideration comes from the observation that coated vesicles formed from large liposomes have a higher buoyant density than small liposomes formed into coated liposomes of a similar size (Figure 6). Thus, the coat is capable of achieving a high density if provided a membrane surface of sufficient size to permit lateral clustering of coat subunits.

Synthetic COPII vesicles form by budding and not by rupture and resealing of membrane fragments. A trapped luminal marker is preserved within the interior of liposomes and isolated COPII vesicles (Figure 8).

The high concentration of COPII protein required to form high-density vesicles and the heterogeneous products of the synthetic budding reaction suggest that additional protein(s) may be necessary to organize the process. An obvious candidate is Sec16p. Sec16 is required for budding in vivo, the gene and mutant alleles display genetic interactions with mutations in other COPII genes, and the protein facilitates the packaging of SNARE proteins into COPII vesicles in vitro (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990; Campbell and Schekman, 1997). Sec16p interacts directly through independent domains with Sec23p, Sec24p, and Sec31p; thus, it may serve to reduce the critical concentration of COPII subunits needed to nucleate the formation of a coat (Espenshade et al., 1995; Shaywitz et al., 1997). In addition, by binding to both heterodimeric complexes

Table 2. Different Concentrations of COPII Proteins Afford the Difference in Sizes of Coated Liposomes Recovered in the High-Density Fraction

	COPII Protein Concentration[a]	Diameter (nm) (Mean ± SD)	Vesicles <90 nm (% of Profiles)	N
Experiment 1	2.5×	54 ± 17	99	240
	5×	85 ± 32	89	240
Experiment 2	2.5×	72 ± 39	94	360
	5×	95 ± 31	82	360

[a] See the legend of Figure 6 for actual concentration of COPII proteins.

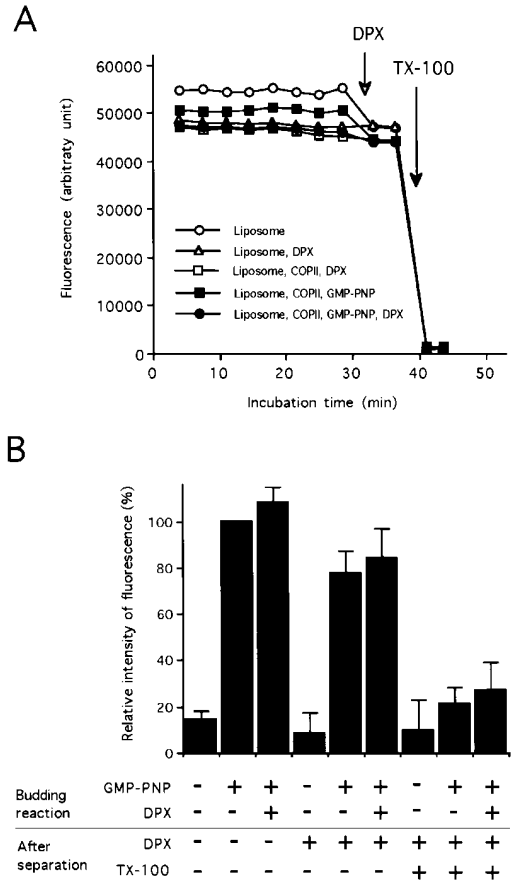

Figure 8. Integrity of Liposomes and Liposome-Derived COPII Vesicles during and after Budding Reaction

(A) Stability of liposomes during the budding reaction. Liposomes that contained the membrane-impermeable fluorescent dye HPTS were incubated at room temperature with combinations of GMP-PNP (0.1 mM), COPII proteins (10 μg Sec13/31p, 8.5 μg Sec23/24p, 8.0 μg Sar1p), and a membrane-impermeable fluorescence quencher, DPX (10 mM), in a 100 μl reaction. The fluorescence of HPTS was monitored during the incubation period. An aliquot of DPX (11 μl of 100 mM) (open arrow) and 2 μl of 20% Triton X-100 (closed arrow) were added to the reaction at the indicated time.

(B) Integrity of liposome-derived COPII vesicles during and after budding reaction. HPTS-containing liposomes were incubated as described in (A). COPII-coated small liposomes were separated by sucrose-density gradient centrifugation as described in the legend of Figure 6A. Fractions 11–15 were incubated with DPX (final concentration 10 mM) or DPX and Triton X-100 (final concentration 0.4%), and the fluorescence of HPTS was recorded. The fluorescence intensity relative to a sample with GMP-PNP but without DPX during budding and after separation is shown. The error bar indicates the standard error of three independent experiments.

of COPII, Sec16p may regulate subunit stoichiometry and ensure a more uniform assembly process.

In spite of the simplicity of the synthetic COPII budding reaction, the parallels with the process reproduced using native ER membranes allow us to draw certain conclusions likely to apply to the physiologic event. The principal and perhaps sole determinant of Sar1p localization to initiate budding is Sec12p, the Sar1p-nucleotide exchange catalyst. This requirement may be bypassed using Sar1p-GMP-PNP, which binds to ER membranes or liposomes made of neutral lipids. Although nucleotide stimulates but is not absolutely required for Sar1p binding, subsequent recruitment of Sec23/24p and then Sec13/31p requires both Sar1p-GMP-PNP and acidic phospholipids (optimally PIP_2 and PI4P) in the liposome. We have suggested that the combination of Sar1-GTP (or GMP-PNP) and Sec23/24p serves to decipher transport signals on SNAREs, on membrane cargo, and on secretory protein receptors (Schekman and Orci, 1996; Kuehn et al., 1998). These membrane proteins may passively participate in the budding process by recruitment into patches created when Sec13/31p completes the formation of the COPII coat.

Phospholipid Requirements for COPII Assembly

Neutral phospholipids promote binding of Sar1p-GMP-PNP to liposomes. Binding is enhanced if the surface hydrophobicity and membrane fluidity is increased with lysophospholipids or free fatty acids. Phospholipids that contain a saturated fatty acyl chain decreased binding probably by decreasing fluidity and increasing the phase transition temperature of the membrane. Because Sar1p has no hydrophobic adducts, we suggest that GTP (or GMP-PNP) induces a conformational change that exposes a hydrophobic surface suitable for partial embeddment of the protein in an exposed bilayer.

The recruitment of Sec23/24p to liposomes requires both activated Sar1p and acidic phospholipids. Among the acidic species tested, low concentrations of PI4P and PIP_2 supported binding of both Sec23/24p and Sec13/31p. Thus, PI4P or the subsequent modification to make PIP_2 could be important for protein transport from the ER. A role for PI4P or PI4-kinase has been suggested in other aspects of the secretory pathway in higher eukaryotes (Liscovitch and Cantley, 1995; Matsuoka et al., 1995). In yeast, PI4P represents around 2.5% of all inositolphosphoglycerolipids (Kaibuchi et al., 1986). However, because inositolphosphoglycerolipids represent only 10–20 mol% of the phospholipids in yeast (Zinser and Daum, 1995), the average concentration of PI4P would be below that necessary to allow COPII binding. Of course, PI4P or PI4-kinase may be enriched in the ER or, specifically, in the ER transitional zone, which is organized for budding from the ER (Orci et al., 1991; Bannykh et al., 1996; Tang et al., 1997).

Yeast cells possess two PI4-kinases. One of them, Pik1p, is a soluble protein located in the cytosolic and nuclear fractions (Flanagan et al., 1993; Garcia-Bustos et al., 1994). At least part of this pool could be associated with the nuclear envelope, which represents an active zone for budding of ER-derived COPII vesicles (Bednarek et al., 1995). Several PI4-kinase isoforms have been identified in mammalian cells, one of which, PIKα, a homolog of yeast Pik1p, is localized to the ER (Nakagawa et al., 1996; Wong et al., 1997). This isoform is unlikely to participate directly in the cell-surface mediated signal transduction process known to involve PI4P. Thus, a role for PIKα in secretion in mammalian cells should be considered.

Unfortunately, it may be difficult to establish unambiguously a role for PI4P or PIP_2 in secretion because other

acidic phospholipids such as PA substitute in our COPII binding assay, albeit at a lower efficiency. Phospholipase D (PLD), which catalyzes the hydrolysis of phospholipids to produce PA, has been implicated in the ER→Golgi limb of the mammalian secretory pathway (Bi et al., 1997). However, a physiological role for PLD in the yeast secretory pathway is uncertain. Yeast cells contain a single PLD (Spo14p) that is related to enzymes in higher eukaryotes but that is not required for mitotic growth and secretion (Rose et al., 1995). No firm conclusion can be drawn from the *spo14* mutant because a distinct Ca^{2+}-dependent PLD has been detected (Waksman et al., 1997) and, alternatively, PA could arise directly through de novo synthesis in the ER.

Given the biochemical evidence for a role of inositol phospholipids in clathrin-mediated vesicle budding and protein sorting (Gaidarov et al., 1996; Rapoport et al., 1997) and the powerful genetic and physiological evidence supporting a role for PI3-kinase in protein transport to the yeast vacuole (Schu et al., 1993), we consider our evidence direct support for a role of phosphorylated inositol phospholipids in the COPII budding event. Other lipids or possibly even proteins may contribute to this process by enhancing the rate of vesicle budding. It remains to be seen if these phosphorylated inositol phospholipids contribute to the protein sorting event that accompanies vesicle budding. We should be in a position to test this by measuring the sorting of representative cargo and resident proteins in the liposome budding reaction described in this report.

Experimental Procedures

Strain and Materials

Saccharomyces cerevisiae RSY445 (*gal2*, *leu2–3*, *112*, *ura3–52*, *trp1–289*, *his4–579*, *prb1*, *pep4::URA3*, *MAT*α) was used for the preparation of ER-enriched microsomes as described by Wuestehube and Schekman (1992).

Most of the phospholipids and derivatives were purchased from Avanti Polar Lipid. PI4P, PIP_2, CDP-DAG, and ergosterol were purchased from Sigma. HPTS, DPX, and SYPRO Red protein stain dye were purchased from Molecular Probes. ^{125}I-Protein A was purchased from ICN, and the ECL Western Blotting System was purchased from Amersham. Antibodies against Sec23p, Sec13p, Sar1p, and Bet1p were described previously (Hicke and Schekman, 1989; Barlowe et al., 1993; Salama et al., 1993, Bednarek et al., 1995).

Sec23/24p and Sar1p were prepared as described (Barlowe et al., 1994). In some cases, Sec23/24p was further concentrated. Sec13/31p was prepared by the modification of Salama et al. (1993) from *S. cerevisiae* RSY1113 (*trp 1–1*, *his3–11,15*, *MAT*α, pNS3141) (Salama et al., 1997).

Preparation of Lipids from ER-Enriched Microsomes and Quantitation of Phospholipids

Lipids were extracted from microsomes with acidic $CHCl_3$/MeOH essentially as described (Matsuoka et al., 1995). Lipids recovered in the organic phase were dried by passing through anhydrous Na_2SO_4 and repeated evaporation with benzene. Dried lipids were dissolved in $CHCl_3$ and stored at −20°C under argon.

Lipids were separated by TLC on an oxalate-impregnated silica gel 60 plate with alkaline solvent as described (Matsuoka et al., 1995). The TLC plate was then immersed in 0.001% purimuline in acetone/water (4:1 by volume) and air dried. Fluorescence of the lipid-purimuline complex was visualized using a STORM 860 image analyzer (Molecular Dynamics). Intensities of the spots of lipids were quantified by ImageQuant software (Molecular Dynamics).

Assay of COPII Binding to Urea-Washed Microsomes and Proteoliposomes

Microsomal membranes were washed with urea as described (Kuehn et al., 1998). For a binding reaction, 2.5 μl of urea-washed membranes were incubated on ice for 15 min with various combinations of COP II proteins and nucleotides in a 50 μl reaction. Each reaction mix was layered on 150 μl B88 (20 mM HEPES-KOH [pH 6.8], 0.15 M KOAc, 0.25 M sorbitol, and 5 mM $Mg(OAc)_2$) containing 0.3 M sucrose and centrifuged at 80,000 rpm in a Beckman TLA 100 rotor for 10 min at 4°C. Proteins in the pellet were separated by SDS-PAGE and transferred to a Millipore Immobilon-P membrane. Sections of membrane were probed with anti-Sec23p, anti-Sec13p, anti-Sar1p, and anti-Bet1p. Membrane-bound antibodies were visualized by ECL or ^{125}I-Protein A. ^{125}I-Protein A–decorated membranes were exposed to a phosphoscreen and analyzed on a PhosphorImager (Molecular Dynamics).

Proteoliposomes were prepared from urea-washed microsomal membranes as described (Brodsky et al., 1993) and subjected to the COPII binding assay.

Preparation of Liposomes and Binding of COPII Proteins

Lipids were hydrated with 20 mM HEPES-KOH (pH 7.0), 0.15 M KOAc, and 0.25 M sorbitol at room temperature with occasional vortexing. The resulting suspension of multilamellar liposomes was extruded through a polycarbonate filter with 400 nm or 50 nm pore size (Poretics) and used for binding experiments. In certain experiments, HTPS (50 mM) was included in the buffer during the preparation of liposomes. Such liposomes were filtered on a Sepharose CL 2B column (Pharmacia) to remove unincorporated HPTS.

For a typical COPII binding experiment, 25 μl of a liposome suspension was mixed with 75 μl of a COPII protein solution containing 2 μg Sec13/31p, 1.7 μg Sec23/24p, and 1.6 μg Sar1p in B88 containing 1.65 M sucrose in the presence or absence of 0.133 mM GMP-PNP. After incubation on ice for 15 min, 75 μl of B88 containing 0.75 M sucrose and 10 μl B88 were overlaid on the incubation mixture, and the resulting step gradient was centrifuged at 100,000 rpm in a Beckman TLA-100 rotor for 90 min at 4°C. Twenty-five microliter samples were collected from the top of the tube. Proteins in the fraction were separated by SDS-PAGE, stained by SYPRO Red and visualized using a STORM 860 image analyzer.

For the two-step binding experiment, the first incubation reaction was scaled up 10-fold as above with appropriate combinations of COPII proteins and liposomes made by a mixture of phospholipid containing 49 mol% DOPC, 21 mol% DOPE, 8 mol% DOPS, 5 mol% DOPA and 8 mol% PI, 2 mol% PI4P, 0.8 mol% PIP_2, 2 mol% CDP-DAG, 2 mol% NBD-PE, and 2 mol% NBD-PC. After incubation, 750 μl of B88 0.75 M sucrose and 50 μl B88 were overlaid on the incubation mixture, and the resulting gradient was centrifuged at 100,000 rpm in a Beckman TLA100.3 rotor for 90 min at 4°C. The top 250 μl of the gradient was collected, and 25 μl of this fraction was used for the second binding reaction.

For the sedimentation analysis, liposomes were made from a mixture containing 80% (by weight) of phospholipids and 20% of ergosterol. The molar ratio of each phospholipid was the same as in the two-step binding reaction described above. NBD-phospholipids were omitted in the phospholipid mixture when HPTS was included in the liposomes. COPII proteins, GMP-PNP (final concentration 0.1 mM), and liposomes were incubated in a 100 μl reaction in B88. After incubation, the reaction mixture was layered over a sucrose gradient and the gradient was overlaid with 200 μl of B88. The resulting gradient was centrifuged for 16 hr at 55,000 rpm in a Beckman TLS55 rotor. Fractions (22 × 100 μl) were collected from the top in a microtiter plate. The fluorescence of NBD-phospholipids or HPTS in each fraction was quantified using a STORM 860 image analyzer. To analyze the integrity of liposomes and coated vesicles in each fraction, we mixed 11 μl of 100 mM DPX with each fraction and monitored fluorescence. Triton X-100 (13 μl of 4% v/v) was then added to each fraction, and the fluorescence was recorded as described above.

To monitor the integrity of liposomes during the budding reaction, we mixed liposomes containing HPTS with combinations of GMP-PNP, COPII proteins, and DPX in 100 μl reactions in wells of a microtiter plate. Samples were incubated at room temperature, and

the fluorescence was recorded using a STORM 860 image analyzer. After a 30 min incubation, 11 μl of 100 mM DPX was mixed with the reaction and the fluorescence was recorded. A final fluorescence measurement was conducted after addition of 3 μl of 20% Triton X-100 to the reaction to dissolve liposomes.

Estimation of the Stoichiometry of COPII Proteins in the COPII Vesicles and Small COPII-Coated Liposomes

COPII-coated vesicles were prepared from microsomes and purified by density gradient centrifugation (Barlowe et al., 1994; Bednarek et al., 1995). Small COPII-coated liposomes in fractions 12 and 13 from the sucrose gradient were applied to a gel filtration column (Bio-Gel A-15m, Bio-Rad Laboratories), and fluorescent material eluted in the void volume was pooled. Proteins in native and synthetic COPII vesicles were resolved by SDS-PAGE and quantified.

Electron Microscopy

Liposomes were made from a mixture of phospholipids and ergosterol by extrusion with a 400 nm polycarbonate filter. The composition of lipids in the liposomes was the same as in the sedimentation analysis. Binding was carried out with liposomes, GMP-PNP, and various combinations of COPII proteins on ice for 15 min in a 250 μl reaction. The resulting mixture was treated with 250 μl 4% glutaraldehyde and 500 μl 2% OsO_4 in cacodylate buffer and incubated on ice for 1 hr. Fixed materials were sedimented by centrifugation at 56,000 × g for 30 min. Pellets were processed for conventional EM as described (Orci et al., 1993b). Sucrose gradient–separated liposomes were fixed with 2.5% paraformaldehyde on ice for 1 hr. The aldehyde-fixed material was further fixed with 1% OsO_4 on ice for 1 hr and processed. COPII-coated vesicles were processed for conventional EM (Barlowe et al., 1994).

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